Higher Frequency of Selective Losses of HLA-A and -B Allospecificities in Metastasis Than in Primary Melanoma Lesions

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Expression of HLA class I molecules is essential for the recognition of tumor cells by $CD8^+$ T cells. In this study, 48 bioptic samples of 42 patients in all stages of melanoma were investigated after short-time cultivation of tumor cells. To confirm melanocytic origin of cultured cells, samples were screened for mRNA expression of melanoma markers gp100, tyrosinase, MAGE-3, MelanA, and MUC18 by reverse transcriptase-polymerase chain reaction. Surface expression of specific HLA-A and -B allospecificities on melanoma cells were analyzed with a standard microcytotoxicity assay after stimulation with interferon (IFN)-α and compared with the background found in peripheral blood mononuclear cells from the corresponding patients. Immunohistochemistry and flow cytometry confirmed specific losses in cases where the appropriate monoclonal antibodies were available. The level of expression of HLA-I, HLA-II, and intercellular adhesion molecule 1 antigens on melanoma cells cultured in the presence or absence of IFN-α and IFN-γ was determined cytofluorometrically. All cell cultures tested were found to be positive for one or more melanocytic markers by reverse transcriptase-polymerase chain reaction. The specific HLA-I alleles on the cultured cells were detectable in 45 of 48 samples. In 11 cases a specific loss of one HLA-I allele was observed (2×A2, B7, B8, B18, 4×B44, B47, B49). Ten of these samples were derived from locoregional lymphnode metastases or from distant metastatic tumors. Only one sample from a primary melanoma showed a specific loss of HLA-I (B47). IFN-α upregulated expression of HLA-I up to 4-fold. IFN-γ enhanced the appearance of HLA-II up to 35-fold and the expression of intercellular adhesion molecule 1 up to 40-fold. Selective loss of HLA-I allospecificities might be a major step in tumor progression. ***Key words: HLA-I/HLA-II/ICAM-1. J Invest Dermatol 111:497–502, 1998***

Efficient specific recognition of malignant cells in melanoma and other neoplasms by the immune system requires surface expression of HLA antigens. Loss or downregulation of these proteins is one of several mechanisms resulting in immune-escape of metastasizing cells (Marincola *et al*, 1994). The expression levels of HLA class I and II on the surface of tumor cells have been examined extensively (Ferrone and Marincola, 1995; Garrido *et al*, 1997) and the following abnormalities were found in melanoma cells: (i) nonfunctional β2-microglobulin (β2M) leads to a total loss of HLA-I restricted recognition by cytotoxic T lymphocytes (CTL) (D'Urso *et al*, 1991), but increases susceptibility to lysis mediated by natural killer cells (Maio *et al*, 1991); (ii) genomic loss resulting in expression of only one HLA haplotype (Marincola *et al*, 1994); (iii) locus specific downregulation due to defects in the transporters associated with antigen processing (Cromme *et al*, 1994; Keating *et al*, 1995; Straten *et al*, 1997); and (iv) selective loss of class I allospecificities, which impairs the ability of a malignant cell to present a specific restriction element to lymphocytes (Kageshita *et al*, 1993a; Rivoltini *et al*, 1995; Luboldt *et al*, 1996).

Most studies, however, have limited the analysis to the monomorphic component of HLA-I (Ruiter *et al*, 1991). Such an approach describes the HLA-I expression as an all-or-none phenomenon and underestimates the frequency of locus and allele-specific aberrations. Furthermore, the analysis of established melanoma cell lines, which have been cultured *in vitro* for many passages in the absence of immunologic pressure, might not represent the expression pattern of HLA moleclules on neoplastic cells *in vivo*. Recently, the availability of monoclonal antibodies (MoAb) specific for single HLA-I alleles has facilitated a more detailed analysis of the HLA status. These studies, however, are limited to the HLA-A1, -A2, and -A3 allospecificities, which show a combined frequency of over 55% in the caucasian population. These allospecificities deserve special interest because several melanoma-associated antigens (MAA) are recognized by $CD8^+$ lymphocytes in the context of HLA-A molecules. These antigens include members of the MAGE family (Zakut *et al*, 1993), gp 100 (Bakker *et al*, 1994), and tyrosinase (Wolfel *et al*, 1994).

Little is known about the time point of the occurrence of HLA alterations in disease evolution. Therefore, we systematically screened short-time melanoma cell cultures for the loss of HLA-A and -B alleles. A selective loss of HLA-I allospecificities was unraveled in 27% of advanced lesions. In primary lesions a selective loss was detected only in one of 11 cases.

HLA-II restricted presentation of antigens is important for the costimulation of CTL against class-I specific tumor antigens. Some

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Abbreviations: β2M, beta 2-microglobulin; CTL, cytotoxic T lymphocyte; ICAM-1, intercellular adhesion molecule 1 (CD54); MAA, melanoma-associated antigen.

MAA are also directly recognized by $CD4^+$ T cells (Topalian *et al*, 1996). Intercellular adhesion molecule 1 (ICAM-1) (CD54) is an adhesion molecule that plays a major role in the attraction of $CD8^+$ effector cells (Becker and Brocker, 1995). Expression of both surface molecules is strongly enhanced upon stimulation with interferon (IFN)-γ, whereas IFN-α only upregulates HLA-I. Therefore we tested the stimulatory capacities of both IFN-α and IFN-γ on melanoma cell cultures. Expression of HLA-II antigens was observed in 9% of all samples, whereas significant expression of ICAM-1 was observed in more than 56% of all cultures tested. These defects might be a critical event for the escape of tumor cells from immunosurveillance.

MATERIALS AND METHODS

Patients Forty-eight surgically removed melanoma lesions were collected under aseptic conditions from a total of 42 patients. Eleven patients suffered from a primary tumor, 15 patients showed locoregional lymphnode metastase and 16 patients had remote secondary tumors, including one sample (no. 39 in **Table I**) from brain-metastases. Melanoma cells were released and dissociated from washed and fragmented tissue sections by serial incubation with dispase (Boehringer, Mannheim, Germany) diluted 1:2 in RPMI1640, and with collagenase (Sigma, St. Louis, MO) diluted 1:100 in tris-buffered saline containing 10 mM $CaCl_2$. The outgrowing cells were subcultivated as described elsewhere (Becker *et al*, 1993). Consecutive metastatic samples from three patients were accessed at 5, 11, and 21 mo, respectively, after the first biopsy. From one patient three melanoma biopsies from different locations and one sample from a later time point were cultured in parallel.

Cell culture Established melanoma cultures were routinely cultivated as monolayers at 37°C and 5% CO_2 in RPMI 1640 (Gibco-BRL, Paisley, U.K.) supplemented with 10% heat-inactivated fetal calf serum (Seromed, Berlin, Germany), 5 mM glutamine (Seromed), 1 mM sodium pyruvate (Gibco-BRL), and 1% of antibiotic mixture containing 10,000 U penicilline per ml and 10,000 μg streptomycine per ml (Gibco-BRL). The cell line UKRV-Mel2 (provided by Dr. D. Schadendorf, Department of Dermatology, Mannheim, Germany) was used as a control and was grown under the same conditions (Schadendorf *et al*, 1996). Confluent, adhering cells were detached with a solution containing 0.05% trypsin and 0.02% ethylenediamine tetraacetic acid (Seromed), inactivated with fresh media and subcultivated once a week.

Polymerase chain reaction (PCR) Total RNA was extracted from 5×10^6 to 10^7 cells using RNeasy kit (Qiagen, Hilden, Germany). Two to four micrograms of RNA were used to synthesize cDNA (M-MuLV reverse transcriptase: New England Biolabs, Beverly, MA). PCR was performed with PCR DIG-labeling nucleotide mix (Boehringer) and with 2.0 μmol oligonucleotide primers per ml. All cDNA were first amplified with primers for β-actin to test for quality and quantity of cDNA. Only satisfactory cDNA was then subject to amplification with primers for tyrosinase, gp100, MUC18, MAGE-3, and MelanA. Primer sequences used for PCR were for tyrosinase cDNA sense 5′ ATG GAA CGC CCG AGG GAC CTT TAC 3′ and anti-sense 5′ TGA GAG GCA TCC GCT ATC CCA GTA 3′, for gp100 cDNA sense 5′ TAT TGA AAG TGC CGA GAT CC 3′ and anti-sense 5′ TGC AAG GAC CAC AGC CAT C 3′ (Adema *et al*, 1994), for MUC18 cDNA sense 5′ CCA AGG CAA CCT CAG CCA TGT 3′ and anti-sense 5′ CTC GAC TCC ACA GTC TGG GAC GAC T 3′ (Hoon *et al*, 1995), for MAGE-3 cDNA sense 5′ CCC AGG CTC GGT GAG GAG GCA 3′ and anti-sense 5′ CTG GTG ACT CGG CAG CAG GCA 3′, and for MelanA cDNA sense 5′ GTG CCC TGA CCC TAC AAG ATG 3′ and anti-sense 5′ CAT AAG CAG GTG GAG CAT TGG 3′. Primers for PCR were designed using OLIGO primer analysis software 4.0 (National Biosciences, Plymouth, MN) and checked on nucleotide sequences published on gene bank by the National Center for Biotechnology Information (Bethesda, MD) to exclude cross-binding (Altschul *et al*, 1990). For all amplifications, one cycle (93°C for 2.5 min, 64°C for 1.5 min, and 72°C for 1.5 min) was followed by 35 cycles (93°C for 30 s, 64°C for 30 s, and 72°C for 1.5 min), and 10 min at 72°C. An aliquot of the PCR product was analyzed by electrophoresis on a 1.6% agarose gel. Bands were visualized by staining with ethidium bromide. In all reactions various cell lines were included as positive controls and H_2O instead of cDNA as negative control. For PCR-enzyme-linked immunosorbent assay, nucleotide probes were selected and checked for specificity as described above, and obtained biotinylated (Microsynth, Balgach, CH). PCR enzyme-linked immunosorbent assay was performed according to kit directions (Boehringer). Hybridization was specific at 45°C for all probes. The specific capture probe/PCR product hybrids were bound to streptavidin-coated microtiter plates via the probes' biotin label. After washing, the immobilized hybrids were treated with anti-DIG peroxidase-conjugated antibody and ABTS, a peroxidase substrate. Absorbance (A492) was measured and values more than twice the negative control were rated positive for expression of the mRNA in question.

HLA phenotyping The original HLA-I and HLA-II phenotype of all patients was established on blood samples collected in acidic citrate dextrose tubes using a complement mediated microlymphocytotoxicity test (lymphotype AB120 and DR60 from Biotest, Dreieich, Germany; Dupont 1987). Frozen peripheral blood mononuclear cells (PBMC) were used if no fresh sample was available. Lymphocytes were separated first with immunomagnetic beads (Dynal, Oslo, Norway) into $CD8^+$ and $CD4^+$ fractions. Melanoma cells were pretreated with 500 U of IFN-α per ml (Roferon, Hoffmann-LaRoche, Basel, Switzerland) for 40 h at 37°C to upregulate surface expression of HLA-I molecules. Cells were harvested as described above and washed once with Hank's balanced salt solution (without Ca^{++}/Mg^{++}/phenolred; Gibco-BRL) prior to HLA-I typization. The same test system was used but incubation times were doubled compared with blood samples. All microcytotoxicity assays were scored at the department of transplantation surgery by the same experienced person to ensure consistent reproducibility and reliability of the test.

Monoclonal antibodies The following primary MoAb were used: W6.32 (ATCC HB-95, mouse IgG2a) reacting with a monomorphic determinant of HLA-I A, B, C molecules; L243 (Becton Dickinson, San Jose, CA; mouse IgG2a; fluoroscein isothiocyanate-conjugated) recognizing human HLA class-II DR epitopes; L368 (ATCC HB-149, mouse IgG1), recognizing cytoplasmic β2M; 6B11 (provided by P. Romero, Ludwig Institute for Cancer Research, Lausanne, Switzerland, mouse IgM, biotinylated) reactive with HLA-A1 allotype (Carrel *et al*, 1994); BB7.2 (ATCC HB-82; mouse IgG2b) reactive with HLA-A2 allotype; BB7.1 (ATCC HB-56; mouse IgG1) reactive with HLA-B7 allotype; 8.4 A6 (Ancell, Bayport, MN; mouse IgG1; fluoroscein isothiocyanate-conjugated) recognizing D2 domain of the human ICAM-1 molecule; SL11.14 (Ancell; mouse IgG2a) recognizing natural killer H1a epitope of the human NCAM (CD56) molecule; Me1–14 (provided by P. Romero, mouse IgG2a) reacting with high molecular weight MAA (Carrel *et al*, 1991).

Flow cytometric analysis To upregulate expression of surface molecules melanoma cells were pretreated with 500 U per ml of either IFN-α (Roferon) or IFN-γ (Rentschler Biotechnology, Laupheim, Germany), for 40 h at 37°C. Stimulated and nonstimulated cells were harvested as described above. Viability and concentration was determined by trypan blue-exclusion assay, only samples with a viability of over 90% were used for further analysis. Cells were washed once with F-PBS (PBS without Ca^{++}/Mg^{++} containing 1% fetal calf serum and 0.02% sodium azide). For cytoplasmic antigens cells were permeabilized in 500 μl of permeabilizing solution (Becton Dickinson) for 10 min at room temperature in the dark. Approximately $5–10 \times 10^5$ cells each were incubated with different antibodies at a concentration of 2–4 μg per 10^6 cells in a total volume of 200 μl for 45 min on ice in the dark. After washing, non-conjugated antibodies were stained with 1:30 dilution (in F-PBS) of fluoroscein isothiocyanate-conjugated rabbit anti-mouse IgG secondary antibody (Dako, Glostrup, Denmark) for 30 min on ice in the dark. Biotinylated antibodies were stained with fluoroscein isothiocyanate-conjugated streptavidin (Dako), diluted 1:50. In all cases, cells were also stained with the appropriate isotype-matched control antibodies, nonspecific mouse IgG1, IgG2a, or IgM (Ancell). After washing and fixation with 0.5% formaldehyde in F-PBS, fluorescence of the cells was measured on a FACSCalibur (Becton Dickinson). Nonviable cells were gated out. Flow cytometric data are presented as mean of fluorescence intensity *versus* cell counts.

Immunohistochemistry Immunohistochemical staining was performed using the APAAP method (Cordell *et al*, 1984). Cryostat sections (≈5 μm) were fixed in cetone for 10 min at room temperature, dried for 2–6 h, and stored at –20°C until further use. Filtered hybridoma supernatants were applied undiluted to the dry sections in a moist chamber and incubated for 1 h at room temperature. Anti-mouse Ig and the APAAP complex (Dako) were added and incubated for 30 min each. The alkaline substrate was applied for 20 min, followed by hematoxylin counterstaining. Between the different steps tissue sections were washed in tris-buffered saline.

RESULTS

Reverse transcriptase-PCR enzyme-linked immunosorbent assay and flow cytometric analysis for melanocytic antigens All cell cultures tested showed mRNA expression of at least one and up to all five of the markers from the test panel, thereby confirming their melanocytic origin. The control cell line UKRV-Mel2 reacted strongly positive with all five markers. Keratinocyte and fibroblast cell lines used as negative controls did not reveal any mRNA expression of the melanocytic markers (data not shown). Of 41 melanoma cell cultures

Table I. HLA typization of blood and cell culture from three groups of melanoma patients and flow cytometric analysis of cell surface markers

No.	Patient	Stage, TNM[a]	HLA-I (PBMC)[b]		Loss[c]	HLA-I[d]	HLA-II[d]	CD54[d]
00	UKRV	nd (cell line)	negative		nd	neg.	10.9	**3.8**
01	950504	I, T3N0M0	A2/3	B7/51	no	2.09	2.6	**17.2**
02	950803	I, T2N0M0	A28/31	B8/60	no	0.98	2.2	nd
03	951010	I, T3N0M0	A29/32	B7/45	no	2.40	2.8	nd
04	951019	I, T3N0M0	A1/28	B44/57	no	3.23	2.2	nd
05	960514	I, T4N0M0	A25/31	B18/27	no	1.95	10.5	**11.4**
06	960704	I, T1N0M0	A1x	B63/47	**B47**	1.49	5.7	**18.2**
07	960724	I, T1N0M0	A2x	B7/51	no	2.01	3.8	12.9
08	961008	I, T1N0M0	A26/30	B35/51	no	1.32	4.3	**20.7**
09	961121	I, T4N0M0	A2/3	B35/38	no	3.81	3.1	**11.0**
10	961122	I, T1N0M0	A2/11	B8/63	no	2.36	7.5	**13.7**
11	961205	I, T3N0M0	A2x	B51/57	no	2.32	12.7	**12.7**
12a	941209	II, TxN2aM0	A1/2	B13/60	no	1.69	1.4	17.9
12b	950822	II, TxN2aM0	A1/2	B13/60	**A2**	1.88	**1.3**	**2.3**
13	950320	II, TxN2bM0	A26/32	B27/44	**B44**	2.94	3.9	7.9
14	950330	II, TxN2bM0	A1x	B13/57	no	2.97	1.4	42.7
15	950622	II, TxN2aM0	A2/3	B7/18	**B18**	1.22	4.6	10.9
16	950710	II, TxN1M0	A2/11	B35/44	no	1.39	0.7	nd
17a	950728	II, TxN2aM0	A11/24	B18/63	no	2.23	8.8	**26.1**
17b	960611	II, TxN2aM0	A11/24	B18/63	no	1.69	1.0	**5.7**
18	960104	II, TxN2bM0	A2/3	B8/44	**B44**	3.18	8.3	22.6
19	960207	II, TxN2aM0	A1/2	B57/60	no	1.66	5.0	nd
20	960312	II, TxN1M0	A1/3	B8/62	no	1.53	3.2	8.1
21	960502	II, TxN2aM0	A26/30	B13/27	no	2.17	2.7	nd
22	960507	II, TxN1M0	A2x	B7/60	**B7**	1.29	3.2	17.8
23	960816	II, TxN2aM0	A29/31	B15/57	no	1.20	3.3	11.0
24	961024	II, TxN2aM0	A1/31	B7/62	no	2.01	4.8	**10.3**
25	961119	II, TxN2aM0	A11/29	B44/51	no	1.79	1.9	34.3
26	961127	II,TxN2bM0	A2/25	B44/58	**B44**	1.77	3.3	**17.5**
27a	950322	III, TxNxM1b	A2/3	B7/51	no	1.30	**1.6**	**6.4**
27b	961209	III, TxNxM1b	A2/3	B7/51	no	2.51	5.8	28.2
28	951004	III, TxNxM1a	A2/3	B13/35	no	1.81	**1.5**	**5.7**
29	960306	III, TxNxM1a	A1/3	B8/62	**B8**	2.48	1.9	2.3
30	960509	III, TxNxM1a	A3/24	B38/51	no	1.75	3.3	nd
31a	960523a	III, TxNxM1a	A1/29	B7/39	us	1.59	5.1	**17.6**
31b	960523b	III, TxNxM1a	A1/29	B7/39	no	2.17	nd	nd
31c	960523c	III, TxNxM1a	A1/29	B7/39	no	1.92	nd	nd
31d	970320	III, TxNxM1a	A1/29	B7/39	no	1.97	9.6	17.1
32	960618	III, TxNxM1a	A2/28	B8/44	**B44**	1.18	2.8	**10.7**
33	960626	III, TxNxM1a	A11/30	B49/51	no	1.88	3.9	**39.4**
34	960819	III, TxNxM1a	A1/2	B7/18	no	2.23	**3.0**	**3.3**
35	960920	III, TxNxM1a	A1/2	B8/62	no	1.47	4.1	17.1
36	960924	III, TxNxM1a	A2/3	B7/49	**B49**	1.08	1.5	**17.2**
37	961023	III, TxNxM1a	A24/30	B51/62	no	1.88	3.2	28.2
38	961213	III, TxNxM1a	A3/11	B37/44	no	2.02	3.4	**10.3**
39	970109	III, TxNxM1a	A2/68	B62x	no	0.88	0.9	4.9
40	970314	III, TxNxM1a	A3x	B7/61	no	2.02	16.8	12.4
41	970604	III, TxNxM1a	A2/25	B44x	**A2**, us	2.31	35.3	**25.9**
42	970715	III, TxNxM1a	A1/32	B52/57	us	1.05	16.5	**15.4**

[a]TNM classification according to UICC (International Union against Cancer).
[b]x, homozygous.
[c]On melanoma cells; us, unspecific reaction.
[d]Samples positive for surface molecules in the absence of interferons are indicated with bold values; data shown as factor of stimulation (mean fluorescence of IFN-stimulated *versus* unstimulated cells); nd, not determined.

tested, 31 were found to be positive for MUC18, 22 for tyrosinase, 20 for MAGE-3, 16 for gp100, and 15 for MelanA. Alternatively, some cell cultures were also stained for high molecular weight-MAA with the Me1–14 MoAb. All melanoma cultures tested were found to be positive for high molecular weight-MAA by flow cytometric analysis.

HLA phenotype of melanoma cells The HLA-I phenotype of all patients is shown in **Table I**. Typization of HLA-II was possible only with fresh samples, frozen PBMC resulted in unspecific basal toxicity, probably due to partial destruction of B lymphocytes during freezing (data not shown). Without incubation with IFN-α the results of the microcytotoxicity assay were not reliable due to the low expression level. The specific HLA-I allospecificities were determined in 45 of 48 melanoma cell cultures after incubation with IFN-α. They were found to be identical to those seen in the corresponding PBMC. Two samples (31a, 42) showed an unspecific basal activity and sample 41 from a heavily pigmented melanoma cell culture was completely nonreactive; however, flow cytometry with MoAb BB7.2 MoAb specific for HLA-A2 revealed a clear loss of this allospecificity. Some cultures were analyzed several times over a period of 4 mo. The results did not change during the cultivation. Probe 12a showed no loss of any HLA-I allospecificities, but in probe 12b from the same patient 8 mo later a clear loss of HLA-A2 was observed (**Fig 3*d***). This loss was confirmed by immunohistochemistry and by cytofluorometry. Probes 17a/17b and 27a/27b, originating from the same patient with an interval of 11 and 12 mo, respectively, resulted in the same HLA-I phenotype. The four probes 31a–d originating from metastases of different locations and a consecutive sample, respectively, of the same patient also showed the same HLA-I phenotype. In general, the HLA-A alleles were easier to determine and were expressed constantly and

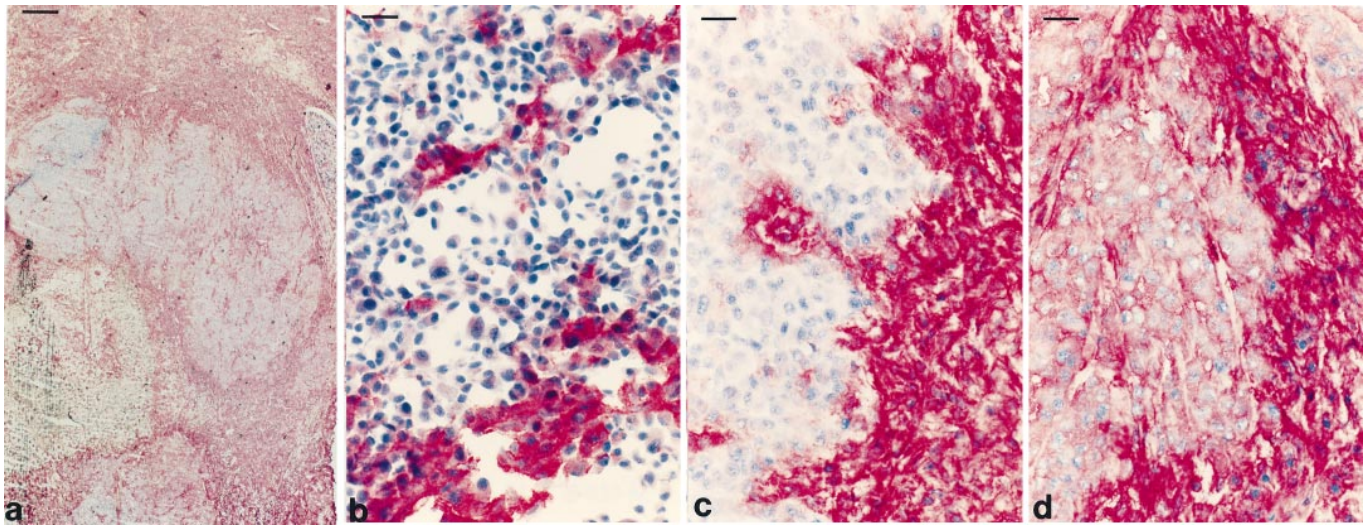

Figure 1. Immunohistochemical staining of cryostat sections from tumor biopsies of melanoma patients. (*a*) Sample 22, with a specific loss of HLA-B7 allospecificity, stained with BB7.1 MoAb; (*b*) sample 12a stained with BB7.2 MoAb specific for HLA-A2; (*c*) sample 12b also stained with BB7.2 MoAb and with W6.32 MoAb (*d*). *Scale bars*: (*a*) 400 μm, (*b*) 50 μm, (*c*, *d*) 25 μm.

homogeneously. The well-characterized cell line UKRV-Mel2 used as a control clearly showed a loss of all HLA-A and -B alleles. HLA-B alleles often showed some minor unspecific side reactions. From a total of 48 samples, 11 patients showed a specific loss of one HLA-A or -B allele in their melanoma cells, 10 of them having locoregional lymphnode metastases (stage II; A2, B7, B18, 3×B44) or distant metastases (stage III; A2, B8, B44, B49). Only one patient with a primary superficial spreading melanoma (Clark level I) presented a HLA-B47 loss. All losses were confirmed by repeated testing. Neither a total loss of complete HLA-I (due to a nonfunctional β2M, as seen in the UKRV-Mel2 control cell line) nor a loss of one haplotype was observed in all samples tested.

Immunohistochemical staining of tumor samples Cryostat sections of tumor biopsies from patients 15 and 22, both typed positive for HLA-B7 from the blood, were stained with the B7-specific MoAb BB7.1. As demonstrated in **Fig 1**(***a***), the whole tumor of patient 22 lacked expression of HLA-B7 surface molecule. The surrounding healthy tissue and intratumoral vessels showed clear immunoreactivity for HLA-B7. When stained with W6.32 MoAb reacting with a monomorphic determinant of HLA-I, tumor tissue was stained, demonstrating expression of other HLA-I allospecificities. Patient 15 presented a heterogenous but clear positive pattern for HLA-B7, this corresponds well with the result from the microcytotoxicity assay, where only two of five wells specific for HLA-B7 showed a positive reaction (data not shown). **Figure 1**(***b***) shows sample 12a, which stained positive with BB7.2 MoAb specific for HLA-A2. In contrast sample 12b did not express any HLA-A2 (**Fig 1***c*), although the other HLA-I allospecifities were present (**Fig 1***d*). Several sections stained with 6B11 (anti-HLA-A1) and BB7.2 (anti-HLA-A2) MoAb showed good correlation with the results from the microcytotoxicity assay (data not shown).

Influence of IFN-α and IFN-γ on surface expression of HLA-I, HLA-II, and ICAM-1 antigens The effects of both interferons are presented in **Table I**. Data are indicated as a factor of stimulation, i.e., mean fluorescence of IFN-treated cells *versus* untreated controls. When compared with appropriate isotype control all melanoma cells were observed to express low to intermediate levels of HLA-I. The control cell line UKRV-Mel2 was totally negative for HLA-I, even in the presence of IFN-α (**Fig 2***a*). This is due to a nonfunctional β2M, as demonstrated with L368 MoAb (**Fig 2***c*); however, expression of HLA-II at a high (**Fig 2***b*) and of ICAM-1 at an intermediate level (data not shown) was observed. In all cell cultures IFN-α upregulated expression of HLA-I up to 4-fold. IFN-γ stimulated expression of HLA-I less efficiently (data not shown). Expression of HLA-II was not detectable in 42 of 46 untreated samples but was upregulated (up to 35-fold the

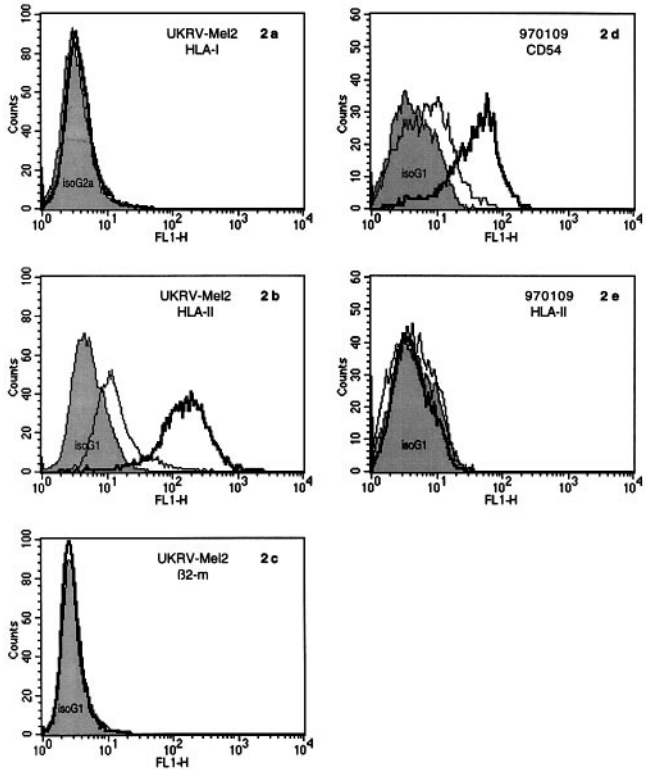

Figure 2. Detection of immunocritical surface molecules on melanoma cells. Cytofluorometric analysis of control cell line UKRV-Mel2 stained for surface expression of HLA-I (*a*), HLA-II (*b*), for cytoplasmic β2M (*c*), and of sample 39 stained with MoAb specific for ICAM-1 (*d*) and HLA-II (*e*). Cells cultivated in the presence of IFN-α (*a*, *c*) or IFN-γ (*b*, *d*, *e*) are shown as bold lines. Isotype control is represented by shaded areas.

value obtained with isotype, mean 5.1 ± 5.8) after stimulation with IFN-γ. In sample 39 originating from a metastasis in the central nervous system, HLA-II did not respond to treatment with IFN-γ (**Fig 2***d*), although ICAM-1 showed the normal stimulation pattern (**Fig 2***e*). This culture also expressed CD56, the neuroneal adhesion molecule (data not shown). Expression of ICAM-1 was enhanced by IFN-γ up to 40-fold (mean 15.8 ± 9.6); without stimulation 17 of 39 tested melanoma cell cultures did not express significant levels of ICAM-1. Over 95% of the tumor cells of sample 12a were clearly positive for

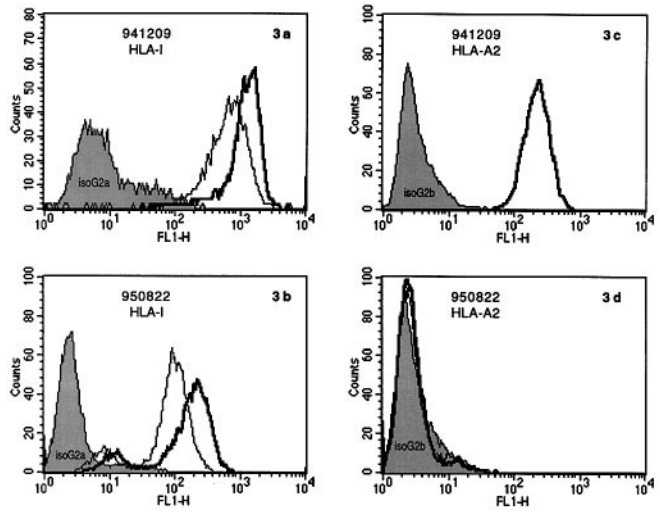

Figure 3. Cytofluorometric analysis of melanoma cells from patient 12 cultured in the absence (fine line) or the presence (bold line) of IFN-α. Samples were stained with MoAb specific for HLA-I (*a*, *b*) or HLA-A2 (*c*, *d*). Isotype control is represented by shaded areas.

HLA-A2 using the monospecific BB7.2 MoAb (**Fig 3*c***), whereas of sample 12b all cells stained negative, even in the presence of IFNα (**Fig 3*d***). This confirmed the specific loss of the HLA-A2 allospecificity detected by the microcytotoxicity assay. Expression of the other HLA-I allospecificities was unaltered in both samples, as demonstrated by staining with MoAb W6.32 (**Figs 3*a*, *b***).

DISCUSSION

The HLA status of a tumor is critical for its recognition and rejection by the immune system. In melanoma, the level of expression of *pan*-HLA-I and *pan*-HLA-II also has prognostic implications (van Duinen *et al*, 1988). A detailed knowledge of the HLA status including allospecifities is a precondition for a successful immunotherapeutical intervention. This is especially true, if peptides, peptide-pulsed dendritic cells (Nestle *et al*, 1998), or genetically engineered tumor cells are used as vaccines.

Analysis of class I allospecificities in melanoma lesions has been hampered by the limited availability of specific MoAb. Most studies have focused on the expression of HLA-A2, because its high frequency facilitates the accrual of large numbers of patients and anti-HLA-A2 MoAb have been available for many years. Several MAA have been recognized by CTL in the context of HLA-A2 antigens (Bakker *et al*, 1994; Kawakami *et al*, 1994; van der Bruggen *et al*, 1994; Wolfel *et al*, 1994). Selective loss of HLA-A2 antigens has been found in ≈30% of melanoma lesions (Kageshita *et al*, 1993a; Jager *et al*, 1997) and in 40%–80% of other solid tumors (Natali *et al*, 1989); however, these data have to be interpreted with caution as they were mainly collected by immunohistochemistry, a method that is not optimal for quantitative determinations of the HLA status.

Restifo *et al* studied melanoma cell cultures obtained from patients undergoing immunotherapy for metastatic melanoma (Restifo *et al*, 1996). Four of 13 (31%) melanoma cell lines lacked functional HLA-I expression as measured by flow cytometry and immunohistochemistry. Northern blot analysis of RNA extracted from these cells revealed normal levels of alpha-chain mRNA but no β2M mRNA or protein. Previous tumor sections obtained from patients prior to immunotherapy were found to be positive for β2M, suggesting that the loss of β2M may be a mechanism whereby tumor cells can acquire immunoresistance during immunotherapy. This effect is compatible with the concept of immunoselection *in vivo* (Lehmann *et al*, 1995).

Marincola *et al* evaluated HLA class I expression in 24 metastatic melanoma cell lines (Marincola *et al*, 1994). Total loss of HLA class I expression was not noted; instead, a variable degree of expression of HLA-B was found that could be upregulated by IFN-γ. HLA-A allospecificities were consistently expressed in all cell types. Loss of allelic expression was noted in two of 14 HLA-A2 (14%) and in one of three HLA-A29 (33%) positive melanoma cell lines and included a full haplotype, which suggests loss of a genomic fragment. A loss of HLA-B allospecificities was not observed.

We have systematically investigated 48 melanoma cell cultures derived from nonselected primary or metastatic lesions, that were analyzed for their complete HLA-A and -B status. We adapted a microcytotoxicity assay that is routinely applied for HLA typing. Because of the low HLA-I expression level, the cultured melanoma cells had to be stimulated with IFN-α to prevent false negative results, probably due to an overexpression of the c-myc oncogene, which specifically downmodulates HLA-I B expression. This effect is frequently observed in human melanoma (Griffioen *et al*, 1995) and can be antagonized by IFN-α.

We found a selective loss of two HLA-A and of eight HLA-B alleles in a total of 37 melanoma metastases, but only one HLA-B loss out of 11 primary tumors. In three cases we confirmed our results by immunohistochemistry and cytofluorometry using monospecific antibodies. We did not detect a complete loss of HLA-I, although the frequency of a total HLA-I loss is estimated to be about 16% in primary lesions (Ferrone and Marincola, 1995). The increased frequency of HLA-B losses in metastases compared with primary tumors suggest that this event has biologic significance during disease progression. In fact, cytotoxic T cell clones are reported that recognize melanoma antigen in the context of HLA-B molecules, although much more HLA-A restricted clones are characterized. This might be due to the frequency of certain HLA alleles, to the limited availability of MoAb against the HLA-B molecules, or to the frequent loss of HLA-B surface expression.

The loss of HLA-B occurred in four of nine HLA-B44 positive patients, but only in one of 11 HLA-B7 and one of seven HLA-B8 patients. These observations propose that HLA-B44 might be an important pathway for the presentation of MAA. It has been shown that specific epitopes of tyrosinase and MAGE-1, −2, and −3 are also presented by the HLA-B44 molecule to cytotoxic T lymphocytes (Brichard *et al*, 1996; Herman *et al*, 1996; Luescher *et al*, 1996). On the other hand, our data are encouraging for vaccination approaches using HLA-A adapted peptides (Jager *et al*, 1996) or dendritic cells pulsed with HLA-I restricted peptides (Nestle *et al*, 1998), that HLA expression remains intact in most of the patients although the density of surface HLA-I molecules on the tumor cells is rather low. This hazard can be opposed by the application of IFN-α that was significantly more efficient in general upregulation of HLA-I than IFN-γ. In all cultures, IFN-γ was able to induce HLA-II and ICAM-1, a surface molecule mediating specific interaction of melanoma cells with CTL (Becker *et al*, 1993; Kageshita *et al*, 1993b; Yue *et al*, 1997). HLA-II restricted presentation of antigens may play an important role in the costimulation of CTL against class-I specific tumor antigens (Rock and Clark, 1996), because $CD4^+$ T cells exist that recognize MAA such as tyrosinase (Topalian *et al*, 1996).

In the adjuvant clinical situation, IFN-α – at least in high doses – has improved the survival of high-risk melanoma patients in contrast to IFN-γ (Kirkwood *et al*, 1996). It may be speculated that this is due to enhanced HLA-I expression by IFN-α. Increased HLA-I expression was found to be correlated with a low relapse rate in melanoma patients (van Duinen *et al*, 1988). Irrespective of the role of class I antigens in the clinical course of the disease, defects in their expression by melanoma cells are likely to have a negative impact on the increasingly applied T cell-based immunotherapy of melanoma (Maeurer *et al*, 1996). *In vitro* data convincingly argue that class I expression in melanoma lesions might represent an important criterion to select patients for T cell-based therapy.

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